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## The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction

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Most of the colour reactions described for formaldehyde require severe conditions incompatible with the preservation of biological material, although consistent results can be obtained where complete destruction of this material can be tolerated (MacFadyen, 1945). Examples of such reactions are those employing chromotropic acid in hot strong sulphuric acid (Eegriwe, 1937), phenylhydrazine and ferricyanide in strong hydrochloric acid (Kersey, Maddocks & Johnson, 1940) and various tests quoted by Walker (1944), some using alkaloids and strong mineral acid and others using phenols and strong alkali. It would be convenient to have at least one colorimetric reaction that could be carried out under less violent conditions. It might also be desirable to preserve biological material on which an estimate of formaldehyde content was required, and from which the taking of samples was inconvenient or impossible.

In the course of work on the neutralization of formaldehyde in living bacterial suspensions, it was found that a yellow colour developed with acetylacetone in the presence of ammonium salts and, less rapidly, in the presence of glycine. This appeared to give a possible alternative to the reactions mentioned above and, as has already been briefly

reported (Nash, 1952), was traced to the formation of 3:5-diacetyl-1:4-dihydrolutidine. Two-stage syntheses of this substance have been described using concentrated reagents and catalysts (Scholtz, 1897; Schneider & Sanger, 1903), but for the particularly simple molecules concerned the reaction can be quantitative for traces of formaldehyde under quite mild conditions.

### *General description of the reaction and its product*

When traces of formaldehyde are added to approximately neutral solutions of acetylacetone and ammonium salt, a yellow colour gradually develops owing to the synthesis of diacetyldihydrolutidine (DDL). Under optimum conditions the molecular extinction in terms of formaldehyde has a smooth maximum of 8000 at 412 m $\mu$ , independent of dilution. The absorption curve is shown in Fig. 1, and was obtained using reagent A (see below); this has since been somewhat modified, but the effect on the absorption curve should be negligible. It can be seen that a good separation is obtained between the absorption maximum of DDL and the first absorption band of acetylacetone. The position of this curve coincided with that obtained from solutions of recrystallized DDL itself in

water, but the molecular extinction of the latter was never greater than 7700. This difference is probably due to changes in the crystals, since after keeping they become more and more difficult to dissolve.

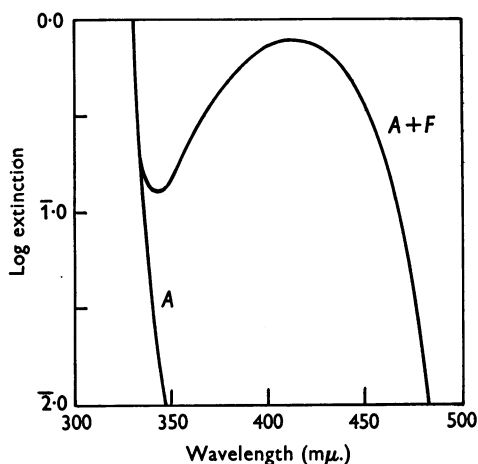
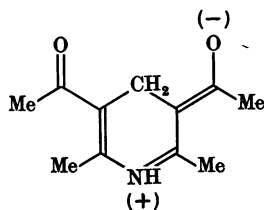


Fig. 1. Extinction due to  $10^{-4}$  M-formaldehyde left standing for 5 hr. at  $20^\circ$  with M-ammonium acetate/0.05 M-acetylacetone/0.03 M-acetic acid (A + F) and the cut-off at the shorter wavelengths due to the control solution (A).

The DDL molecule is probably polar, both in solution and in the crystals. The structure (a) accounts satisfactorily for its insolubility in ether,



its high melting point (about  $200^\circ$ ) and the negligible effect of pH changes on the extinction, over the range 4–10. The two original acetylacetone

molecules can be seen to be linked by the formaldehyde methylene at the top and the ammonia nitrogen at the bottom. One of the possible mesomeric structures is shown; the net charge separation is probably a good deal less than unity.

#### Effect of pH and reagent composition

The reaction at low formaldehyde concentrations appears to be most nearly quantitative at pH 5.5–6.5. In one experiment with ammonium phosphate buffer kept molar in ammonia, and with 0.01 M-acetylacetone, the relative yields measured on a colorimeter were: pH 4, 75 %; pH 5, 94 %; pH 6, 100 %; pH 7, 96 % and pH 8, 87 %. The effect of varying the reagent composition is shown in Table 1, which gives the efficiency as a percentage of that obtained with reagent B (see below). The approximate times for 99 % of the actual reaction at any composition are also given, deduced from absorptiometer readings by assuming a first-order law for the final stages of the reaction, i.e. that the rate of colour development is proportional to the amount remaining to be developed. Owing to fading, estimates for the longer times are not possible, but such solutions would not be generally used. It can be seen that the efficiency falls off much more rapidly with decreasing ammonia concentration than with decreasing acetylacetone concentration. In fact, high yields of DDL can be obtained with only traces of acetylacetone and of formaldehyde, provided that sufficient ammonium salt is present. The practical range, however, can be seen to be restricted to ammonia concentrations between M and 0.1 M and acetylacetone concentrations between 0.1 and 0.001 M.

#### Preparation and use of reagent

It was found that colour production was slightly greater from acetate solutions than from phosphate. Since acetate solutions are usable in the presence of calcium and magnesium salts, if necessary, the following mixture should be of general use

Table 1. Concentrations of diacetyldihydrolutidine reached in 24 hr. at  $20^\circ$  in solutions of different compositions, but with the same initial amount ( $10^{-4}$  M) of formaldehyde

(Slight excess of acetic acid was present, to the extent of 0.05 mol. prop. of the ammonium acetate throughout. Figures are percentages of the value obtained with the standard solution containing M-ammonia and 0.01 M-acetylacetone. Where possible the estimated times, in hours, to attain 99 % of the maximum colour developed, are given in parentheses.)

Strength of acetylacetone	Strength of ammonium acetate				
	3 M	M	0.33 M	0.1 M	0.033 M
0.1 M	99 (3)	97 (4)	94 (16)	81	44
0.033 M	103 (3)	100 (4)	97 (14)	88	48
0.01 M	103 (7)	100 (5)	100 (13)	90	51
0.0033 M	102 (11)	101 (8)	98 (15)	89	53
0.001 M	98 (16)	98 (16)	95	79	45
0.00033 M	74	88	77	61	35

(analytical grade reagents, redistilled acetylacetone, which should be colourless):

*Reagent B*

2M-ammonium acetate or 150 g. approx.  
0.05M-acetic acid or 3 ml.  
0.02M-acetylacetone or 2 ml. } to 1 l. water.

An earlier mixture, referred to as reagent *A*, contained five times as much acetylacetone, but this was found to be unnecessarily strong. Some of the results described below were obtained with reagent *A*, but the difference is negligible; the main reasons for reducing the acetylacetone concentration were that the zero absorption would be less (see below on sensitivity), that the reagent should keep longer (see below on stability) and that the reagent should be less toxic to living material.

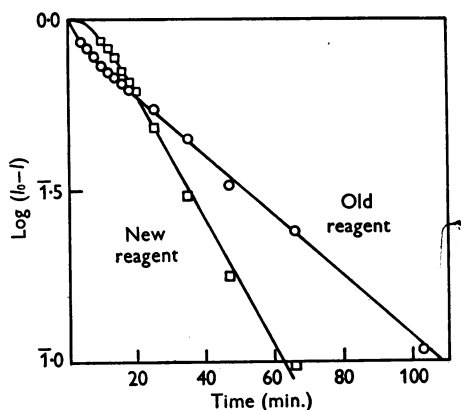


Fig. 2. The development of colour with time using freshly made ammonium acetate-acetylacetone reagent, and 1-day-old reagent, on addition of  $10^{-4}$ M-formaldehyde. Colour development plotted as a first-order reaction;  $I_0$  is the maximum colour developed,  $I$  is the colour at a given time.

The reagent is mixed with an equal volume of solution containing not more than  $8 \mu\text{g.}$  of formaldehyde per ml. (if there is more, DDL will eventually precipitate in long, yellow, flexible crystals) and the colour estimated on a photoelectric colorimeter with a deep-blue filter. From Table 1 the time for 99% reaction is 5 hr. at  $20^\circ$ . To a first approximation the reaction can be treated as unimolecular (Fig. 2), and in fact the times for 99% completion, calculated from unimolecular plots, follow the Arrhenius relation closely (Fig. 3). The reduction in the time required for 99% completion at higher temperatures can then be estimated; for example, reagent *B* at  $37^\circ$  should take only 40 min. and at  $58^\circ$ , 5 min. The shape of the early part of the reaction curve is also changed as the temperature is raised (Fig. 4).

If the reaction is considered for the detection, rather than the estimation, of formaldehyde, it

should be noted that all the components of the reagent are volatile, and that DDL crystals are strongly fluorescent under soft ultraviolet light.

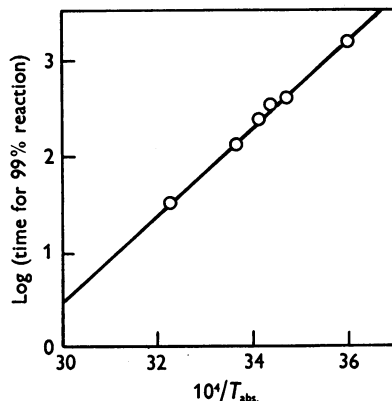


Fig. 3. The temperature coefficient of the reaction between acetylacetone, ammonia and formaldehyde between  $5^\circ$  and  $37^\circ$ .

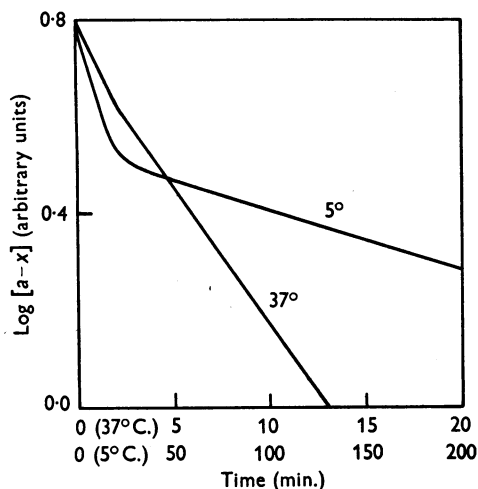


Fig. 4. Rate of production of colour from acetylacetone ( $0.05\text{M}$ ), ammonium acetate ( $\text{M}$ ) and formaldehyde ( $10^{-4}\text{M}$ ) at two different temperatures. Initial stages, using 1-day-old reagent, plotted as a first-order reaction;  $a$  is the maximum final colour developed,  $x$  the amount at any given time.

*Stability of reagent*

The addition of strong ammonium acetate to solutions of acetylacetone causes a slow shift of the edge of the first ultraviolet absorption band towards the visible. This shift has stabilized in 24 hr. at about  $18 \text{ m}\mu$ . from its original position, the solution still remaining colourless to the eye. The extent to which the shift has proceeded at intermediate times has no effect on the final absorption in the blue caused by the addition of formaldehyde,

but merely affects the shape of the reaction curve (Fig. 2). It was found that after 2 weeks at 20°, reagent *A* developed a feral odour and readings from it had dropped to 92 % of those from fresh reagent. Reagent *B*, on the other hand, appeared to be unchanged.

#### *Sensitivity*

For a given amount of formaldehyde the extinction in the present method is only one-third of that obtained in the chromotropic acid reaction at 570 m $\mu$ . (MacFadyen, 1945). However, this is not a true measure of comparative sensitivity because the absorption of control solutions is higher for chromotropic acid. MacFadyen (1945) states that it is between 9 and 25 % for analytical grade reagent. This was confirmed in this laboratory, and even after a long and tedious purification it was still 4 %/cm. On the other hand, reagent *B* has a control absorption of only 1 %, which does not increase appreciably for a week or so if the reagent is protected from traces of formaldehyde. When long light paths are possible, reagent *B* is probably about as sensitive as chromotropic acid.

#### *Measurement of colour*

The calibration curve from absorptiometer readings is not convertible to a straight line by plotting log. intensity of transmitted light against concentration. This is not due to any detectable departure from Beer's Law but is a consequence of the inefficiency of the blue filters available (D. G. Anderson, personal communication). For instance, two kinds of filter tested here both had a noticeable transmission in the infrared, to which the photocell was somewhat sensitive and which was strongly emitted by the tungsten source.

#### *Toxicity*

Tests with a coagulase-negative staphylococcus (N.C.T.C. 2561) showed that the organism was able to grow in peptone water saturated with DDL and a dilution series showed that that growth was at least as good in DDL solution as in one containing an equal mol. prop. of formaldehyde. The dilution of reagent necessary for good growth was adequate for colour development, which agrees with figures given by Schales (1951) for the toxicity of acetylacetone to other bacteria.

#### *Specificity*

Owing to the mild conditions not many substances interfere, especially if the reaction time is kept to the minimum necessary for say 99 % completion. The following have been tested.

Acetaldehyde gives rise to diacetyldihydrocollidine, with an absorption peak of similar intensity to that of DDL but at 388 m $\mu$ . instead of 412 m $\mu$ . The rate of formation is such that about 1 % interference

is to be expected from the same mol. prop. of acetaldehyde as formaldehyde. The reaction would not be suitable in the presence of large amounts of acetaldehyde.

Acetone, chloral, fural and glucose do not interfere. Ninhydrin probably does not if pure; one sample tested showed a small constant absorption equivalent to 0.005 mol. prop. of formaldehyde.

Amines can compete with ammonia in the Hantzsch reaction, though generally the rate of reaction is much less. It was found that 0.1M-methylamine caused 4 % loss, and 0.1 M-ethylene-diamine 20 % loss, of determined formaldehyde. Hexamethylenetetramine is gradually decomposed, yielding 80 % of its formaldehyde on heating with reagent *A* for 2 hr. at 85°. Glycine does not interfere unless very concentrated.

Polymers of formaldehyde react slowly, probably by release of monomer. Paraformaldehyde gives a fairly rapid, but only partial, reaction. There appears to be at least one volatile polymer present in commercial 40 % formalin which is completely unreactive, and which causes estimates of vapour concentration over formalin solutions to come out several times greater if measured by chromotropic acid, than with the present method. On the other hand, good agreement was obtained between the present method and Romijn's iodine method, as applied by Blair & Ledbury (1925) in similar estimations.

Periodate destroys the colour, but this can be prevented by the addition of excess iodine, followed immediately by excess thiosulphate.

Sulphites prevent the reaction in quite low concentration. 0.001M sodium sulphite causes 90 % loss of determined formaldehyde. No work has been done on the reactions involved.

#### *Stability of colour*

Strong oxidizing agents, particularly nitrous acid, destroy DDL either by complete breakdown of the molecule or by oxidation to the colourless diacetyl-lutidine. Daylight causes fading, probably aided by dissolved oxygen, but long exposure is necessary. In one test there was a 3 % loss on standing in the dark in an open test tube overnight, which increased to 25 % after standing all day in front of a window, but away from direct sunlight. A similar solution in a stoppered bottle showed no loss in the dark over 2 days.

### EXPERIMENTAL

#### *Preparation of diacetyldihydrocollidine and three related substances, and a comparison of their ultra-violet absorption*

Acetylacetone, ammonia and formaldehyde are the simplest stable substances that can take part in a Hantzsch reaction. Because of this the reaction or its product might

Table 2. *Optical properties of cyclic compounds*

(For meaning of code names see Experimental section.)

Code name	Colour in daylight	Fluorescence (soft ultraviolet)	$\epsilon_{\max.}$ ( $\times 10^{-3}$ )	$\lambda$ ( $\epsilon_{\max.}$ ) ( $m\mu$ .)
HH/Me	Strong-yellow	Yellow	7.7	412
HMe/Me	Pale-yellow-green	Pale-green	8.4	388
HH/OEt	Straw	Pale-green	6.6	370
HMe/OEt	None	Strong bluish white	7.8	353

Table 3. *Analyses of cyclic compounds*

(For meaning of code names see Experimental section.)

Code name	M.p. (°)	M.p. (literature) (°)	Carbon (%)		Hydrogen (%)	
			Found	Calculated	Found	Calculated
HH/Me	208 (decomp.)	198	67.7	68.4	7.78	7.72
HMe/Me	154	152-153	62.8	63.0	7.86	7.87
HH/OEt	178-180	174-176	62.1	61.7	7.50	7.51
HMe/OEt	129	127-129	69.0	69.6	8.04	7.86

be expected to show some exceptional properties, and to establish the colour reaction on a sound basis it was desirable to find out if this was so or not. It was known from recent work (Haley & Maitland, 1951) that acetaldehyde and ammonia react with both acetylacetone and acetoacetic ester under suitable mild conditions. In view of the DDL results and the general reactivity of formaldehyde, it was reasonable to suppose that ammonia, acetoacetic ester and formaldehyde would also react in a similar manner.

nium salt was desirable, and the concentrations chosen for the preparations were: molar ammonium acetate, 0.2M- $\beta$ -diketone and 0.1M-aldehyde. Solutions were made up as follows. Diacetyldihydrolutidine (HH/Me): 10 g. acetylacetone, 3.75 ml. 40% formalin; diacetyldihydrocollidine (HMe/Me): 10 g. acetylacetone, 3.05 g. acetaldehyde-ammonia; diethyl 1:4-dihydrolutidine-3:5-dicarboxylate (HH/OEt): 13 g. acetoacetic ester, 3.75 ml. 40% formalin; diethyl 1:4-dihydrocollidine-3:5-dicarboxylate (HMe/OEt):

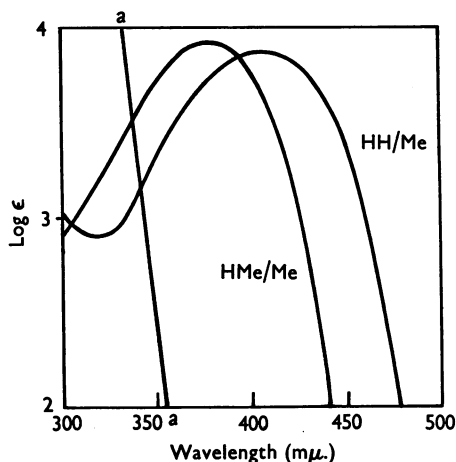


Fig. 5. Molecular absorption of 3:5-diacetyl-1:4-dihydrolutidine (HH/Me) and 3:5-diacetyl-1:4-dihydrocollidine (HMe/Me): *a-a* represents the cut-off by the generating solution.

It was therefore decided to prepare the end products of the four possible Hantzsch reactions between ammonia and formaldehyde, acetaldehyde, acetylacetone and acetoacetic ester. Preliminary experiments showed that reasonable yields were obtained in every case by using ammonium acetate as the only buffer for one-stage preparations in aqueous solution. It was also found that excess of ammo-

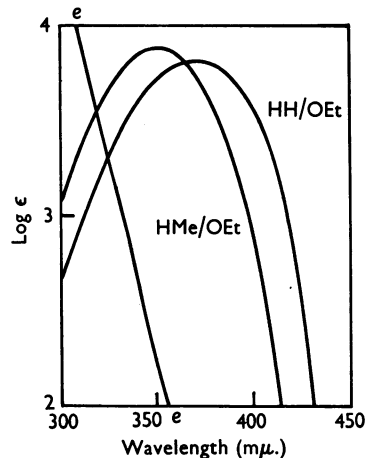


Fig. 6. Molecular absorption of diethyl 1:4-dihydrolutidine-3:5-dicarboxylate (HH/OEt) and diethyl 1:4-dihydrocollidine-3:5-dicarboxylate (HMe/OEt): *e-e* represents the cut-off by the generating solution.

13 g. acetoacetic ester, 3.05 g. acetaldehyde-ammonia. In addition, each solution contained 38 g. of ammonium acetate and was made up to 500 ml. The solutions were allowed to stand at 20-25° for 4 days and the precipitates filtered off and dried. Yields of crude product were: HH/Me, 6.7 g. or 77%; HMe/Me, 4.05 g. or 39%; HH/OEt, 9.0 g. or 71% and HMe/OEt, 11.1 g. or 83%.

The simple code names adopted refer to the different substituents in the basic ring system, HH referring to the ring methylene, HMe to the ring methyl-methylene, Me to the methyls of the acetyl groups and OEt to the ethoxy groups of the ester side chains. See also Figs. 5 and 6.

One recrystallization from ethanol was sufficient to give a product whose melting point agreed with that given in the literature, but up to three more were required before the colour of the crystals did not change further. There was also a marked increase in the fluorescence, particularly in the last compound (HMe/OEt). The melting points were also raised slightly, particularly the first compound (HH/Me, or DDL). These are given in Table 3, together with the analyses. As found by others (Hantzsch, Knoevenagel, quoted by Schiff & Prosio, 1895) there was a general deficiency in the carbon content. Repeat analyses on HH/Me gave figures for carbon of 66.7, 67.7 and 68.6% (averaged for Table 3), while two using a copper tube for combustion gave 69.4 and 69.7%.

**Absorption spectra.** It is clear from Figs. 5 and 6 that the compound HH/Me (DDL) is in no way exceptional. The strong-yellow colour is due to the absorption extending sufficiently far into the visible region; only the last compound, HMe/OEt, was finally obtained as completely

colourless crystals, the two intermediate ones showing some absorption in the extreme blue. The optical properties are summarized in Table 2.

### SUMMARY

A colour reaction for formaldehyde is described which depends on the synthesis of diacetyldihydro-lutidine from acetylacetone and formaldehyde in the presence of excess of ammonium salt. The conditions are mild enough to allow of its use with living material, and its sensitivity and degree of specificity are comparable to those of other reactions requiring more severe conditions.

The work arose from investigations carried out on behalf of the Public Health Laboratory Service Committee on Formaldehyde Disinfection. I am indebted to Dr A. S. McFarlane and to Dr S. Jacobs, of the National Institute of Medical Research, for the use of a spectrophotometer, and to Dr J. Walker, also of the National Institute, for the micro-analyses.

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## Studies in the Biochemistry of Micro-organisms

### 90. ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER, METABOLIC PRODUCTS OF *ALTERNARIA TENUIS*

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Although species of *Alternaria*, in the Dematiaceae family of moulds, are of common occurrence and of world-wide distribution, little work had been published until recently on their biochemical activities. Brian, Curtis, Hemming, Unwin & Wright (1949) reported that several strains of *A. solani* (E. and M.) Jones and Grout and one strain of *A. porri* (Ell.) Sacc. produce, in a variety of liquid media and in fair yields, a metabolic product which they named alternaric acid. This substance, which is not appreciably antibacterial, has a remarkably

specific antifungal activity and is phytotoxic. The same workers and Jefferys (Brian *et al.* 1951) determined the optimum conditions for the production and isolation of alternaric acid and gave a more detailed account of its antifungal properties. They showed that while germination of the spores of some fungi, e.g. *Absidia glauca* and *Myrothecium verrucaria*, is prevented by 1 µg./ml. of alternaric acid, concentrations as high as 100 µg./ml. are required to prevent the germination of the spores of other fungi, e.g. *Botrytis allii*, although, with this fungus,